DOCKET NO: 247232US23

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF	:
DAVID TACHA	: EXAMINER: GRUN
SERIAL NO: 10/784,163	:
FILED: FEBRUARY 24, 2004	: GROUP ART UNIT: 1641
FOR: IMMUNOASSAY REAGENTS AND METHODS OF USE THEREOF	:

DECLARATION UNDER 37 C.F.R. § 1.132

COMMISSIONER FOR PATENTS
ALEXANDRIA, VIRGINIA 22313

SIR:

David Tacha, Ph.D., a citizen of the United States of America, hereby declares and states that:

1. I have a doctorate in Medical Technology.

2. I am and have been employed by Biocare Medical, LLC. since August, 1997and I have a total of 11 years of work and research experience in the field of antibodies, immunochemistry, and related biomedical applications.

3. I am a named inventor on this application and am therefore familiar with the specification and current claims that will be filed with my Declaration.

4. I understand that the U.S. patent office has rejected the claims of this application as being obvious based on what is described in van der Loos (1999, Immunoenzyme Multiple Staining Methods), the specification on page 2, Myers (J. Surg. Pathol. 1:105, 1995) or Hasui (J. Histochem. Cytochem 51:1169,2003) and these citations with U.S. patent no. 6,537,745

(Chien), U.S. patent no. 4,690,890 (Loor), U.S. patent no. 5,108,896 (Philo), U.S. patent no. 5,089,423 (Diamandis) and U.S. patent application 2002/0173053 (Damaj).

5. I have read and understand the contents of these publications as well as those publications cited previously by the U.S. patent office in the communication of March 23, 2007, i.e., Mason et al (J. Can Res. Clin. Oncol. 101, 13, 1981) and Shi et al (Appl. Immunohistochem. Mol. Morph 7:201, 1999).

6. van der Loos generally describes detecting two antigens (starting at page 13) and immunoenzyme triple staining (Chapter 8, starting at page 63). As cited in the communication of March 23, 2007, van der Loos also describes the use of polymer conjugates, but those polymer conjugates are dextran polymer conjugates (referred to as DAKO Envision™) on pages 4 and 15. This, however, is not what has been defined in the claims, nor what is currently defined in the claims. As is apparent in the claims, a secondary antibody is coupled to a poly (alkaline phosphatase (AP)) moiety and/or poly (horseradish peroxidase (HRP)) moiety. These poly (AP) and poly (HRP) are different from the Dextran based technology that van der Loos describes (see also page 12, lines 14-16 of the application, referencing the previously cited Shi et al paper).

7. Indeed, using the very same protocols that are described by van der Loos, the ability to do simultaneous double stains is hampered and not conventional, even as of 2006. For example, DAKO, a company that is also in the business of immunohistochemistry, explains in a lab manual double stains (attached) to do sequential staining (referencing Van der Loos) -see *Conclusion* section. Thus, I do not understand how what van der Loos describes provides any indication as to how to successfully perform a simultaneous double stain in the way as is defined in the application and claims of the patent application.

8. The Meyers paper does discuss automated staining using at least two antibodies, but based on page 109 and table 2 of Meyers the method used is a sequential application of the antibodies and not a simultaneous double-stain protocol, through a 137-step sequential protocol. Hasui also discusses automated staining protocols. Neither Meyers or Hasui describe employing a secondary antibody is coupled to a poly (alkaline phosphatase (AP)) moiety and/or poly (horseradish peroxidase (HRP)) moiety.

9. Further, nothing in van der Loos, Meyers, and Hasui provides any indication as to how to perform a double stain in not more than 2 to 2.5 hours; nor that a double stain could be performed in not more than 15 steps.

10. Still further, none of these publications describe a primary rabbit monoclonal antibody.

11. Although not cited in the communication of October 18, 2007, the Shi paper was cited before by the patent office in the communication of March 23, 2007. The Shi paper is also referenced in page 12, lines 14-16 of the application.

12. The Shi paper does describe the same poly-HRP/AP conjugates as in the claims of this application. The poly-AP had not been developed or was it commercially available Of course, as previously recognized by the patent office, the Shi paper does not describe a multiple staining protocol, nor how to achieve that. The discussion in Shi certainly noted the sensitivity increase and detection efficiency using such polymer conjugated antibodies and the possibility that these polymer conjugates could be useful in multiple staining protocols, what we achieved in terms of a dramatically reducing the amount of steps required, provide time savings, have vastly better sensitivity than had ever been reported before for multiple staining protocols and also providing the ability to perform automated immunoassays was not at all predictable from what is described by Shi, van der Loos or the other publications cited by the patent office.

13. I have performed countless experiments proving the validity of the method described and claimed in the application. I have also supervised such experiments being performed.

14. A few exemplary experiments are described and shown below. The experiments and attached photographs (Figs. 1-3) compare the type of HRP technology that Mason and van der Loos would have used versus our poly-HRP technology as provided in the claims. The comparisons used the same dilutions, reagents, and protocol except for the actual HRP conjugates. In short, what these data show is the vastly superior results obtained when employing the poly-HRP compared to other conventional HRP conjugation, including those described by van der Loos and Mason.

15. The methodology employed for these experiments is as described in the application, generally, with the following steps.

1. Deparaffinize slides and hydrate to water
2. Block endogenous peroxidase with 3% Hydrogen Peroxidase for 5 minutes.
3. Block non-specific background with protein blocker for 10 minutes.
4. Incubate primary antibody for 30 minutes.
5. Incubate with goat anti-mouse-HRP/rabbit-AP for 30 minutes or goat anti-mouse-AP/rabbit-HRP for 30 minutes
6. Incubate with DAB for 5 minutes.
7. Incubate with Fast Red for 10 minutes
8. Counterstain with Hematoxylin

16. The results for three different double staining protocols are provided in the attached Figures (1-3) with the conventional HRP on the left panel and the poly-HRP in the right panel. As is quite apparent from these photographs, the results, our technology is far superior to a standard HRP technique.

17. The sensitivity of the method coupled with the reagents used, allows us to easily cocktail up to 4 antibodies without producing background staining and further extended to 5 or 6 antibodies with reduced background compared to what had been done before. Indeed, we have, on numerous occasions, performed experiments that show that the method works

well simultaneous triple and quadruple staining (i.e., using 3 or 4 antibodies). Further, the manner in which the claimed method is performed enables one to perform a double stain in not more than 2 to 2.5 hours nor that a double stain could be performed in not more than 15 steps and particularly relevant for the clinical setting, in an automated system that provides fast, clean and very good results. None of the publications describe any of these nor how to achieve such results.

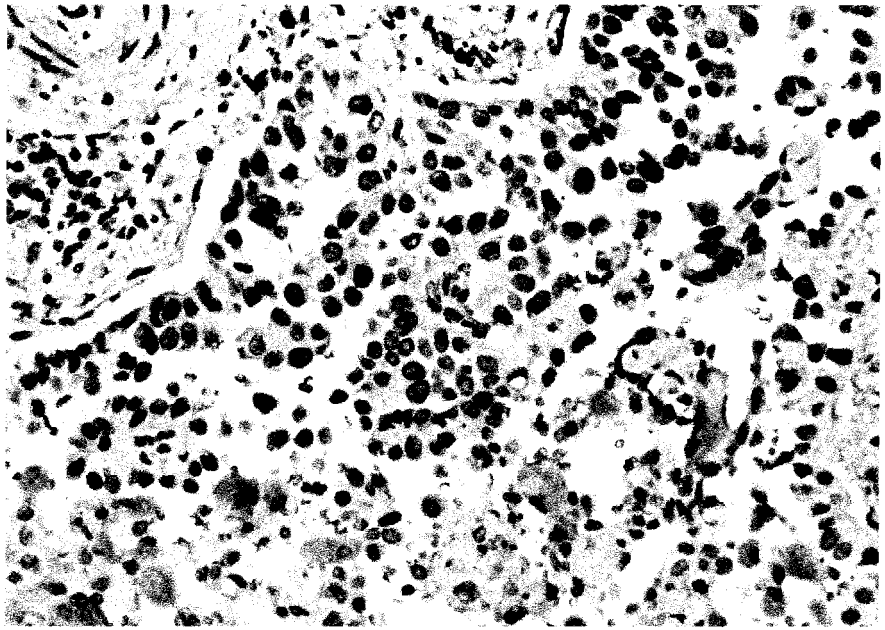
18. While I am not a patent attorney, nor do I have any legal training, it is my view as a scientist with extensive experience in this field, that what we have achieved as exemplified in the figures attached and described in the patent application simply could not have been predicted to work as well as it did based on what was known before the patent application was filed and, particularly, in view of what is described in van der Loos, Myers, Hasui, Shi or the other materials cited by the patent office.

19. I also understand that the patent office has taken the position that the application does not describe the method sufficiently so as to apply more than two primary and/or secondary antibodies and then detect the antigen-antibody complexes so formed. However, as I described above, we have successfully performed triple and quadruple staining using the protocols described in the application. We have tested up to 5 antibodies in one cocktail using these same protocols with nothing more than routine experiments and optimizations.

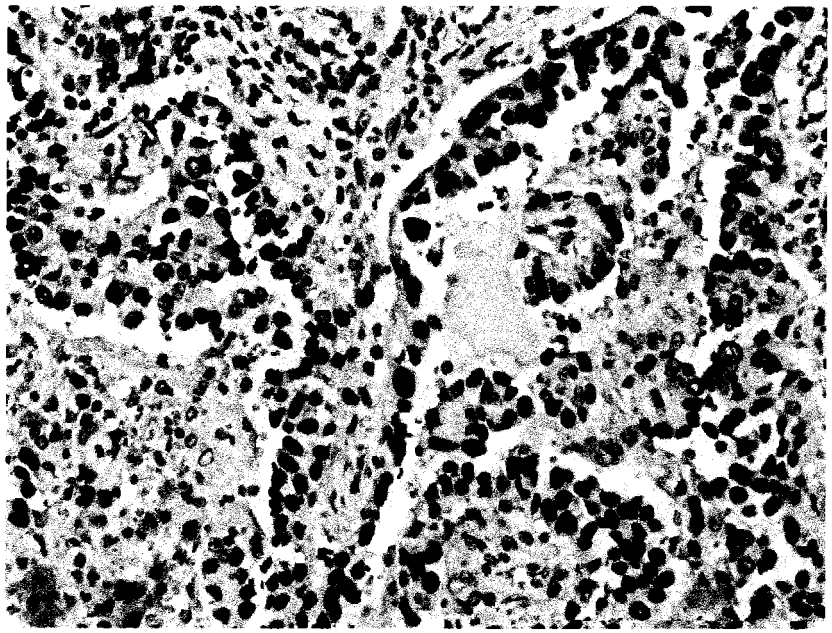
20. I declare under penalty of perjury under the laws of the United States of America that to the best of my knowledge and belief the foregoing is true and correct.

Date: October 6, 2008 David Tacha

David Tacha, Ph.D.

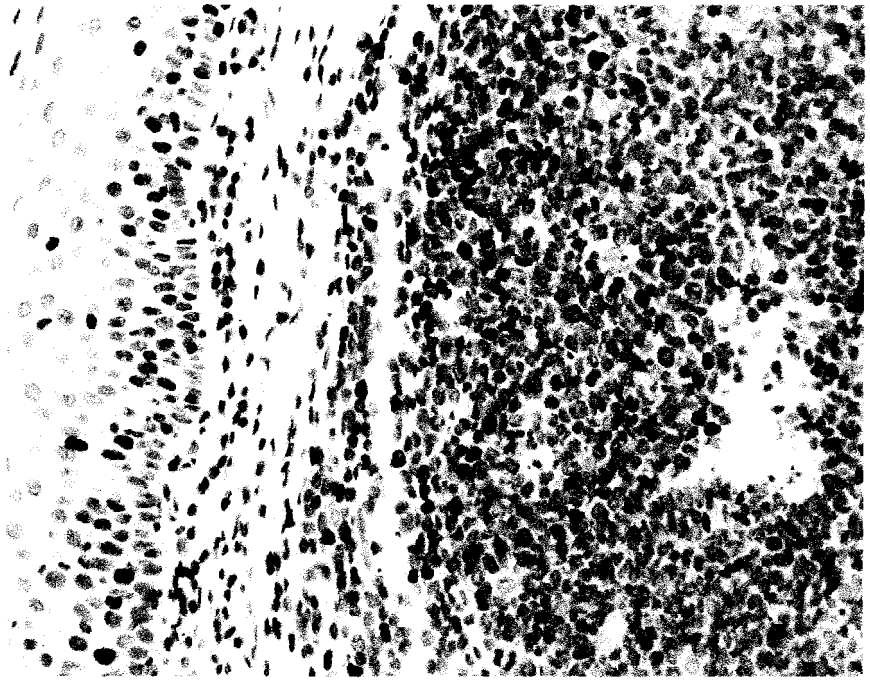


TTF-1 Old HRP Technology

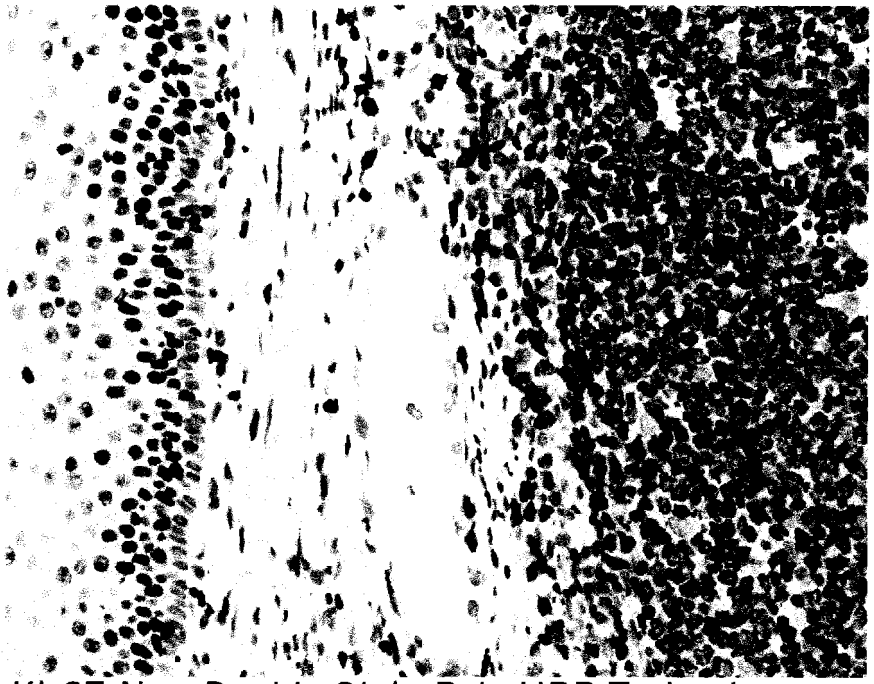


TTF-1 New Double Stain Poly-HRP Technology

FIG. 1

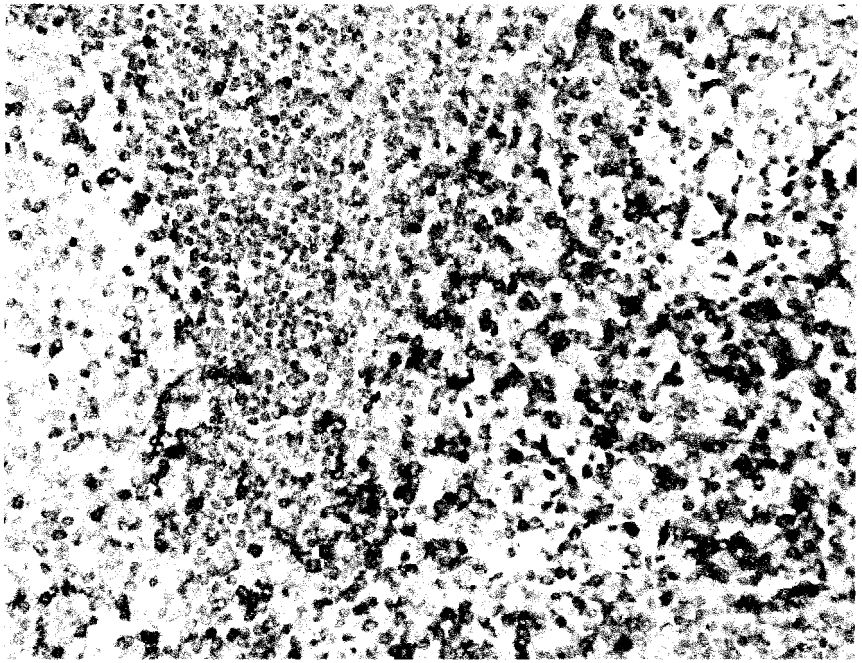


Ki-67 Old HRP Technology

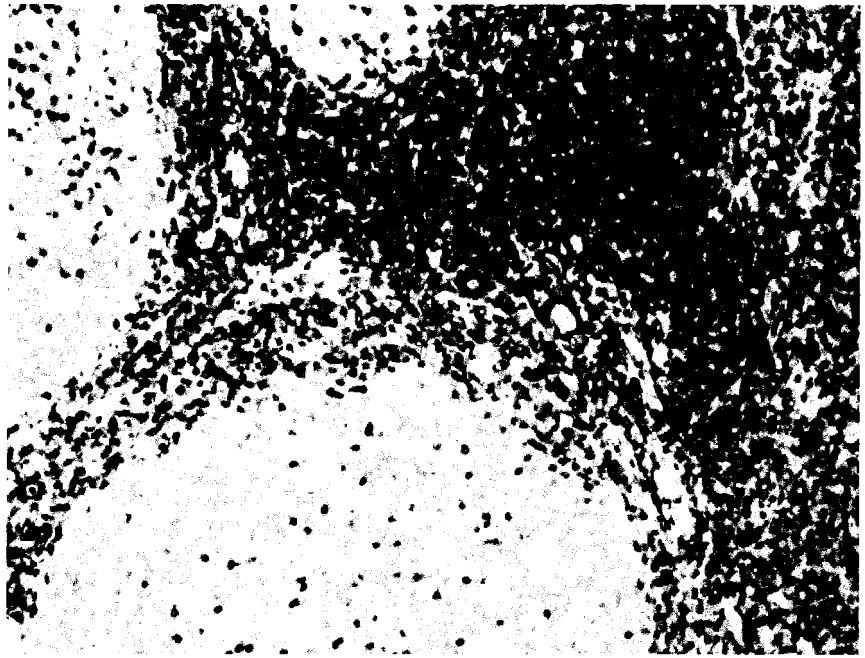


Ki-67 New Double Stain Poly-HRP Technology

FIG. 2



CD3 Old HRP Technology



CD3 New Poly-HRP Technology

FIG. 3

Dako

PATHOLOGY

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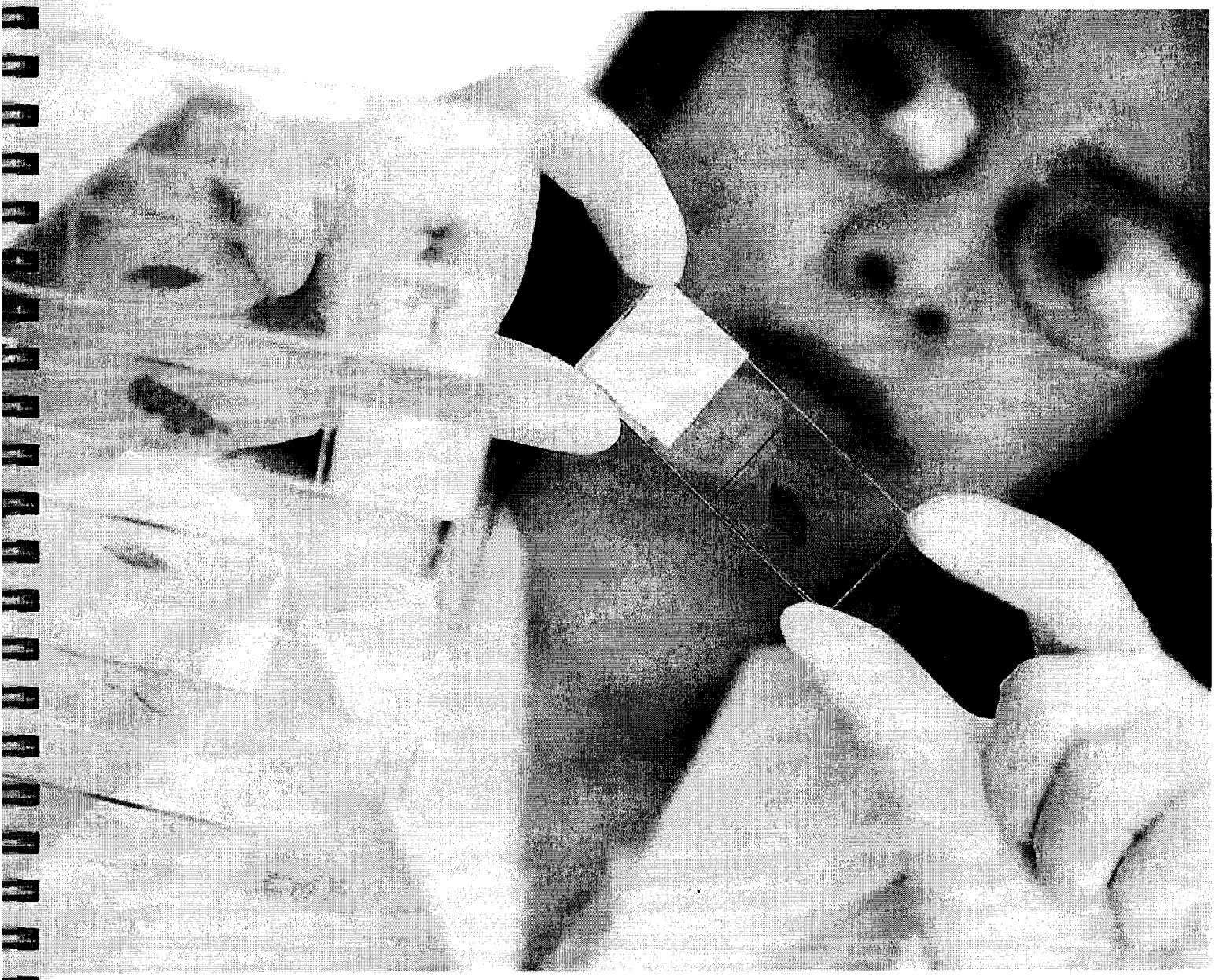

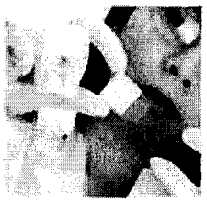

Immunohistochemical Staining Methods, Fourth Edition

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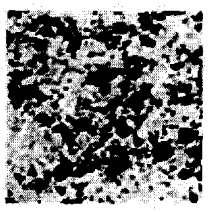


Chapter 9 – Multi-Staining Immunohistochemistry

Nanna K. Christensen and Lars Winther

Introduction

Immunohistochemistry (IHC) has become established as an important tool for both research and diagnostic purposes. However, in some cases there is a need for knowledge about the relative localizations of targets, which can be obtained only by visualizing all relevant targets on one slide. This chapter describes the advantages of multiple staining, as well as the considerations that have to be made to ensure successful staining. This article will discuss the choice of appropriate protocols as well as the choice of visualization systems.

Advantages of Multiple Staining

Multiple staining can be defined as the detection of two or more targets on one slide, thus increasing the information obtained from each slide. Hands-on time per slide depends on the method used. Sequential staining does not reduce hands-on time compared to combining single staining; whereas simultaneous staining does reduce turn-around time (see below). With increasing demand for reduced turn-around-time, multiple staining may offer at least part of the solution. Furthermore, there is a demand for less invasive sampling techniques giving smaller and fewer specimens and available slides. In such cases multiple staining also may be a great advantage.

Equally important, multiple staining makes it possible to assess the topographic relationship of the targets, for example, to determine whether targets are present in different cells, in the same cell or even in the same cellular compartment. Information also can be obtained on possible cell-to-cell spatial contacts of different cell types. Some of this information also can be obtained using single staining on serial sections. However, this is laborious and time consuming and the sections must be very thin to ensure all structures or cells are present in the entire series of sections. Multiple staining allows the combination of in situ hybridization (ISH) and IHC, giving information about a particular target both at protein level and DNA/mRNA level.

The diagnosis of prostatic epithelial neoplasia (PIN) is just one example of the clinical importance of multiple staining. Prostatic needle biopsy is the preferred method for diagnosing early prostate cancer. But in some cases an ambiguous diagnosis is made due to the fact that the biopsy has identified only a few malignant glands or several histological benign mimics of cancer (1). Since basal cells are present in the benign cancer mimics but absent in the malignant glands, these cells can be used to distinguish between the two cases. Basal cells are labeled using high-molecular-weight cytokeratin, cytokeratin 5/6 or p63 immunostaining. In addition, the gene product of p504s, alpha-methylacyl-CoA-racemase is expressed in a high percentage of prostate carcinomas, but is negative or only weakly expressed in benign prostate tissue. Thus it is used as

a positive cancer marker. In cases of small foci, ambiguous lesions may disappear when using serial sections, causing suspected malignancies to remain undiagnosed. A multiple staining protocol reduces the percentage of residual ambiguous lesions and the need for additional biopsies.

Multiple staining is well known from flow cytometry, where staining of three to four different targets on the same cell is routine. A single sample elicits considerable information, allowing unambiguous separation of different cell-types and identification of abnormalities. ISH routinely uses multiple staining on slides to determine gene amplification from the ratio of the signals from the gene probe of interest to a reference probe.

ISH also can be used in multiple staining to detect chromosome translocations using split-signal FISH. Probes directed towards stretches immediately upstream and downstream of the breakpoint are labeled green and red, respectively. Thus when probes are co-localized, the mixture of green and red results in a yellow signal, but when the chromosome breaks, the signals separate and individual green and red signals can be seen. In this case, single-target staining would not give the desired information. Multiple staining is well established in ISH, and there are obvious advantages to extending this to an IHC format to gain the benefit of the additional information.

Technical Challenges

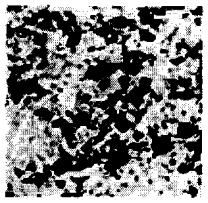
Users experienced with the challenges of single-target staining will find the demands for multiple staining are similar but more complex.

Before embarking on a multi-staining project, some important issues should be considered:

- To avoid target or species cross-reactivity, complex protocols may be necessary.
- Spectral differentiation of stain colors may be difficult, especially if the targets are co-localized. Rare targets that are co-localized with more abundant targets may not show, and if colors are mixed, results may be difficult to separate from single colors.
- Even if targets are not co-localized it is difficult to balance signals enabling rare targets to be visible in the same slide as highly abundant targets. This is due to the narrow dynamic range of IHC. An adjustment in concentration of the primary antibodies may solve this problem.
- If different targets are viewed under different magnifications, it may not be possible to get the topographic information desired.

Pretreatment

Multiple staining, like single staining, can be performed on both formalin-fixed, paraffin-embedded tissue, cryosections, cell smears and cytospin preparations. Multiple staining is constrained by the fact that it may not be possible to find one tissue pre-treatment



protocol that is optimal for all targets. Often protocols optimized for individual staining differ from one target to the other; for example, different target retrieval methods are used. If that is the case, it is necessary to determine a method that allows all targets to be stained, although the method may be sub-optimal for some targets.

In cases where targets of different abundances are to be stained, a method must be selected that gives the best balancing of the signals. Combining ISH and IHC on one slide is challenging, particularly because targets require very different pre-treatment protocols. Since ISH processes such as DNA denaturing are not compatible with the presence of the antibodies for IHC, the ISH protocol normally is performed first.

Staining Method Selection

To ensure success, IHC staining must be planned carefully. This is even more important with multi staining. If primary antibodies are commercially available both directly labeled and unlabeled and from different host-species, there are several different staining methods to choose from; however, very often the choice is limited by the available reagents (2). Care must be taken to avoid cross-reactivity between reagents. A flow chart or similar aid might prove useful in selecting the best method.

In general, staining methods can be divided in the following classes:

Sequential Staining

An indirect technique using unlabeled primary antibodies where the staining of one target is completed including the application of the chromogenic dye, before the application of the next.

The primary and secondary antibodies from the first staining are eluted before staining the next target (for an example, see Figure 9.1). This avoids cross-reactivity-related problems; however, elution may be difficult with some high-affinity primary antibodies, leading to spurious double stained structures. This technique therefore is not recommended for evaluation of mixed colors at sites of co-localization. Elution also risks denaturing epitopes of antigens that are to be subsequently visualized. Furthermore, for some chromogens there is a risk that the first chromogen (DAB in particular) shields other targets. Not all reaction products are capable of surviving the rigorous washing required to remove the antibodies. Thus in order to avoid blurry staining results the most robust dyes should be applied first.

Simultaneous Staining

A direct method with directly labeled primary antibodies, or an indirect method based on unlabeled primary antibodies raised in different host species, or of different Ig isotype or IgG subclass (3).

A simple example of this method is when the primary antibodies are fluorescently or enzyme labeled to allow direct visualization. This avoids cross-reactivity but is rarely

practical since some form of amplification is necessary to get sufficient signal. In that case, primary antibodies are conjugated directly with enzymes, biotin, haptens or fluorochromes, subsequently employing the corresponding antibody or streptavidin reagent as the second layer. This is less time-consuming than the sequential method, since primary and secondary antibodies can be mixed together in two incubation steps. However, it requires avoiding all cross-reactivity.

With the indirect method it is also possible to apply timesaving antibody cocktails. Generally it is advantageous to use secondary antibodies raised in the same host in order to prevent any unexpected inter-species cross-reactivity.

Multi-Step Technique

An indirect/direct method combining unlabeled primary antibodies with antibodies that are directly conjugated. The method starts with the staining of the unlabeled antibody/antibodies.

Sequential staining avoids the problem of cross-reactivity but cannot be used for co-localized targets. The technique often leads to a long staining protocol and carries an inherent risk of incorrect double staining due to insufficient elution of one set of reagents before application of the next Simultaneous staining is less time-consuming since the reagents of each layer can be mixed together. However, the technique can be used only if suitable primary antibodies are available. Multi-step staining can be used when the selection of primary antibodies is limited. However, when using this method, it is not possible to mix reagents.

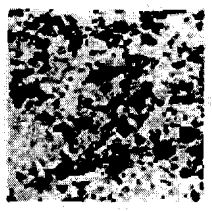
Users often will find that the choice of staining method is limited by the availability of the primary antibodies with respect to species origin or label.

When targets are known or suspected to be co-localized and the only available primary antibodies are unlabeled monoclonal mouse antibodies of the same IgG subclass, none of the techniques described above are applicable.

One solution is the Dako Animal Research Kit (ARK™) Peroxidase, which contains reagents for labeling mouse primary antibodies with a biotinylated anti-mouse Fab fragment, followed by blocking of the remaining reagent with normal mouse serum. This can be applied to the tissue as part of the multi-step technique (4). The kit gives a non-covalently labeled antibody, thus avoiding the risk of reducing the affinity. In addition, only small amounts of primary antibody are needed and the kit does not require time-consuming purification steps.

Another solution is Zenon Technology (Invitrogen) developed for flow cytometry. It uses essentially the same technique, and offers labeling kits for mouse primary antibodies available as enzyme conjugates or conjugated to one of a wide variety of fluorescent dyes.

Visualization systems with dual recognition such as the EnVision™+ Dual Link system do not discriminate between species, and are thus only suitable for multiple staining when



using the sequential method. Visualization kits with amplification layers that are not well specified should be avoided, since possible cross-reactivity cannot be predicted.

Selection of Dyes

The primary choice to make when deciding how to make the targets visible is whether to use immunoenzyme staining or fluorescence. Both have advantages and disadvantages and in the end, decisions should be made based on conditions of the individual experiment.

Chromogenic Dyes

When selecting color combinations for multiple staining with chromogenic dyes, it is advisable to choose opposing colors in the color spectrum, to facilitate spectral differentiation. If using a counterstain, this also must be included in the considerations. When working with co-localized targets, dyes must be chosen so that it is possible to distinguish the mixed color from the individual colors. Double staining using chromogenic dyes is well established, but it is demanding if the targets are co-localized. For triple staining, it is more difficult to get colors that can be unambiguously differentiated and it is very difficult if targets are co-localized.

A narrow dynamic range is a handicap for immunoenzymatic staining. The precipitation process, which is crucial for this method, is only triggered at a certain threshold concentration of substrate/product. On the other hand, at high concentrations the precipitated product may inhibit further reaction. Therefore it is difficult to visualize rare targets and highly abundant targets in the same slide. To reduce this problem using the strongest dye to stain the most rarely expressed target is advantageous. Or if possible use extra amplification like the Catalyzed Signal Amplification (CSA) System to bring rare targets within the same dynamic range as highly expressed targets.

There is a limited range of chromogenic dyes to choose from. These are examples of enzyme/chromogen pairs suitable for triple staining:

- GAL/XGAL/Turquoise, AP/Fast blue, HRP/AEC/Red;
- HRP/DAP/Brown, GAL/XGAL/Turquoise, AP/Fast red;
- HRP/DAP/Brown, AP/New Fucsin/Red, HRP/TMB/Green.

In conclusion, chromogenic dyes can be used successfully for double staining; however, identifying co-localized targets may be a problem. Triple staining is also possible, but great care must be used in selecting dyes.

Fluorescent Dyes

Double immunofluorescence labeling is also quite well established (5). Some of the same considerations as with chromogenic dyes apply when working with immunofluorescence. It is equally necessary to select dyes with distinguishable spectral properties. However, there are more colors available and the emissions spectra of the fluorescent molecules are narrower than the spectra of the chromogenic dyes. DAB in particular has a very broad spectrum. The use of multiple fluorescent colors is also already well established in FISH and flow cytometry, where dichroic filters and bandpass filters are employed to separate different fluorescent signals. The spectral separation can be aided by digital compensation for overlapping emission spectra.

When staining targets that are co-localized fluorescent dyes, allow separate identification of targets. This makes it possible to discern targets even in widely different concentrations, whereas subtly mixed colors may pass unnoticed easily with immunoenzyme staining.

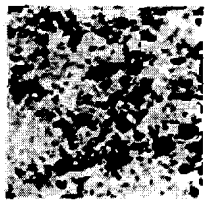
Immunofluorescence potentially has a wider dynamic range than immunoenzyme staining. Using this method, there is no enzymatic amplification involved and thus the dynamic range is determined solely by the sensitivity of the detectors.

On the other hand, there are some inherent problems with the use of immunofluorescence:

- Fluorescence signal is quenched when the fluorochromes are in close proximity.
- Dyes undergo photo bleaching when subjected to light and will thus only fluoresce for a limited time unless stored in the dark.
- Even when stored protected from light, some fluorochromes will deteriorate slowly at room temperature.
- The morphology viewed in slides is different from what is observed in immunoenzyme staining with counterstains.
- Increased background due to autofluorescence can pose a problem when working with some formalin-fixed tissues.
- Leaching of stored antibody conjugates may pose a problem.

In spite of these drawbacks, immunofluorescence gives clear, sharp localization of targets and has advantages over chromogenic dyes when working with co-localized targets.

Some chromogenic dyes fluoresce as well, such as Fast Red, an AP-substrate that is brighter in fluorescence microscopy than in bright field microscopy.



Other Labels

Colloidal gold-labeled antibodies were developed originally for electron microscopy, but with silver-enhancement they are visible with normal light microscopy.

Another example of inorganic stains is Quantum dots (Q-dots). These are fluorescent nanoparticles with varying emission wavelengths, depending on the size of the particle. They can be linked to antibodies or streptavidin as an alternative to fluorochromes (6). However, the size of their conjugates may pose diffusion problems.

Automated Image Acquisition and Analysis

Digital image analysis will increase the number of usable dyes since it does not rely on the human eye for detection and differentiation. A digital image is acquired at excitation wavelengths relevant for the dyes applied, and separate detectors record individual colors. So, for example, digital image analysis will allow the combination fluorescent and immunoenzyme dyes.

Detectors, however, have biased color vision. They amplify colors differently than does the human eye. Therefore dyes used on image analysis should be optimized for the best possible fit with the detector's filter properties.

Image analysis systems contain algorithms that allow compensation for overlapping emission spectra comparable to flow cytometry. They also allow signal gating within an interesting range of wavelengths, enabling users to see only signals within the desired range. Visualizing a combination of several gates with color selected independently of the dyes used for staining may clarify pictures and make conclusions easier to reach. This also makes it possible to set a threshold on signal intensity to exclude unspecific staining or background from final images.

Another advantage of digital image analysis is that it allows signal quantitation. Through software manipulation users, can count how many signal clusters exceed a certain level of intensity, and, potentially, calculate the ratio of different cell-types. For example, an image analysis algorithm can calculate the percentage of cells that stain positive for a certain target, combine that percentage with information of other stained targets and, based on this, highlight diagnosis.

Conclusion

Multiple-target staining will one day be as routine as single-target staining is today.

Use of the technique will expand, since it offers reduced turn-around time and information not obtainable from single-target staining. Availability of labeled primary antibodies, antibodies raised in different host species and multiple staining kits also is likely to increase.

Software for automated image acquisition and analysis will play a key role in this evolution since the limit to how many colors the human eye can distinguish will be

reached soon. Analysis algorithms will never entirely replace a skilled pathologist, but algorithms will improve gradually as the amount of information loaded into underlying databases increases. Eventually algorithms will become sufficiently "experienced" to be able in many cases to suggest a diagnosis, and only the final decision will be left for the pathologist.

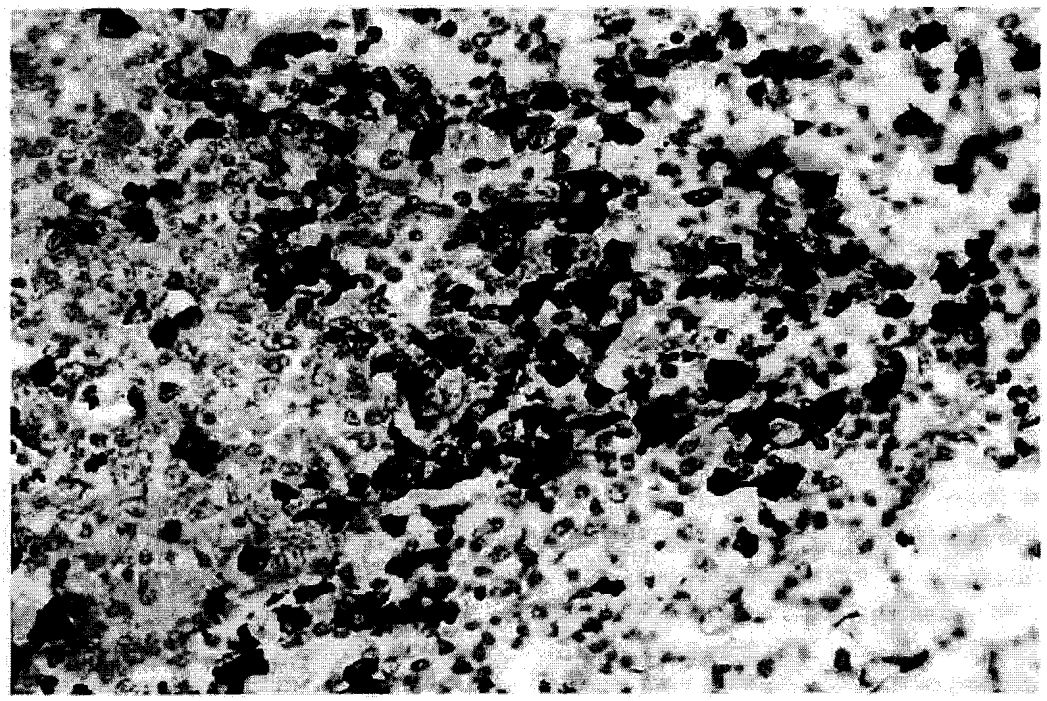


Figure 9.1. Sequential doublestaining method performed with the EnVision™ G|2 Doublestain Kit * using polyclonal anti-kappa (red) and polyclonal anti-lambda (brown) as primary antibodies. Formalin-fixed paraffin-embedded section from tonsil.

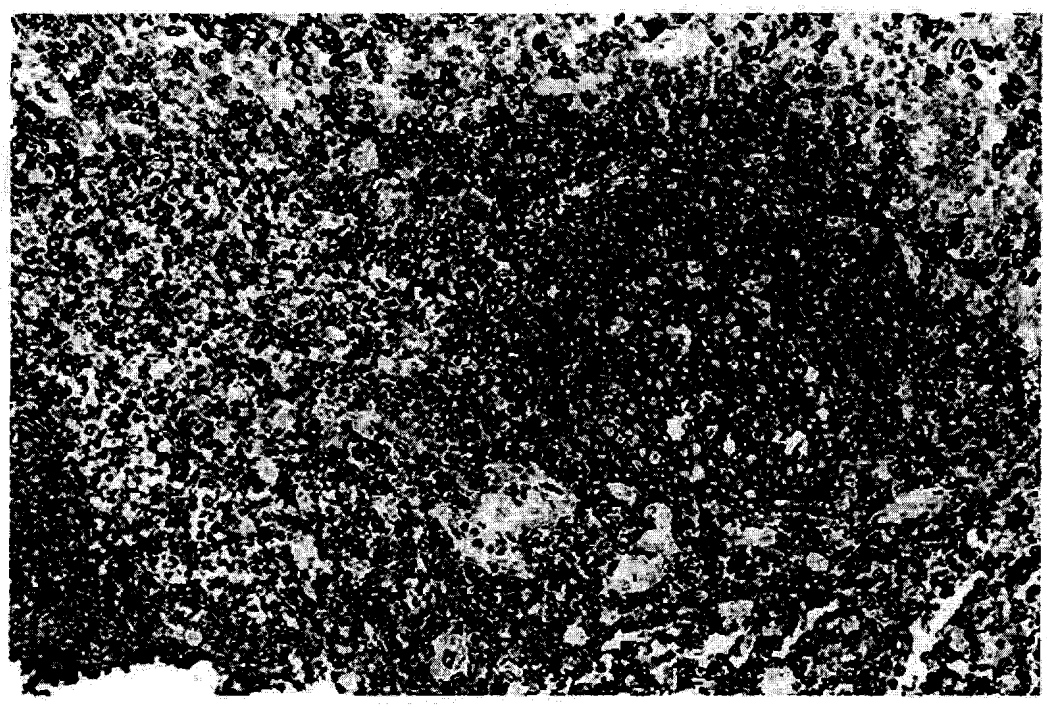


Figure 2. Sequential doublestaining method performed with the EnVision™ G|2 Doublestain Kit * using monoclonal anti-CD3 (red) and monoclonal anti-CD20 (brown) as primary antibodies. Formalin-fixed paraffin-embedded sections from tonsils.

References

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Footnote

* A proprietary methodology developed by Dako.